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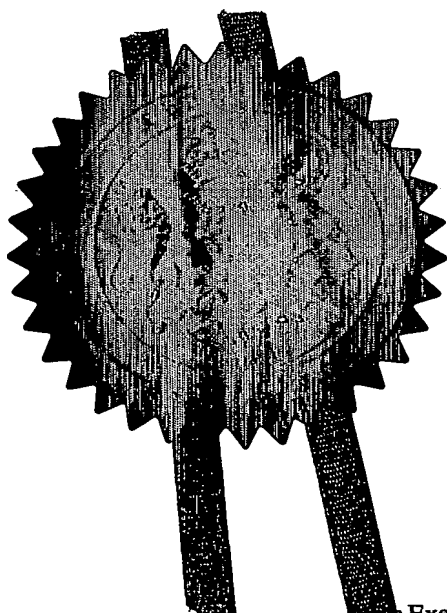
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*Andrew Benz*

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1.	Your reference	N-33276P1	27NOV03 E855255-4 000245 0177700 0.00-0327527.8
2.	Patent application number (The Patent Office will fill in this part)	0327527.8	26 NOV 2003
3.	Full name, address and postcode of the or of each applicant (underline all surnames)	UNIVERSITY OF BERN MURTENSTRASSE 35 3010 BERN SWITZERLAND	
	Patent ADP number (if you know it)	8761298001	
	If the applicant is a corporate body, give the country/state of its incorporation	SWITZERLAND	
4.	Title of invention	Organic Compounds	
5.	Name of your agent (If you have one) "Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	Craig McLean Novartis Pharmaceuticals UK Limited Patents and Trademarks Wimblehurst Road Horsham, West Sussex RH12 5AB 07181522002 ✓	
6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number	Country	Priority application number (if you know it)      Date of filing (day/month/year)
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day/month/year)
8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. (see note (d))	Yes	

## Patents Form 1/77

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Continuation sheets of this form

Description 20

Claim(s) 4 *DL*

Abstract 1

Drawing(s)

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*) 1

Request for substantive examination (*Patents Form 10/77*)


Any other documents  
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11.

I/We request the grant of a patent on the basis of this application

Signature

Date



Craig McLean

26<sup>th</sup> November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr. Trevor Drew

01403 323069

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N-33276P1

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### Organic Compounds

The present invention relates to plant derived material with bone resorption inhibiting activity.

The most common metabolic bone disorder is osteoporosis. Osteoporosis can be generally defined as the reduction in the quantity of bone, either from the reduction in bone formation or the acceleration of bone resorption, in either event the result is a decrease in the amount of skeletal tissue. Osteoclasts (bone resorbing cells) are responsible for the excavation of a portion of bone during the resorption process. After resorption, osteoblasts (bone forming cells) appear, which then refill the resorbed portion with new bone.

In young healthy adults, the rate at which the osteoclasts and osteoblasts are formed and operate maintains a balance between bone resorption and bone formation. However, as normal consequence of aging, an imbalance in this remodeling process develops, resulting in loss of bone. As imbalance continues over time, the reduction in bone mass and thus bone strength leads to fractures.

Many compositions and methods are described in the medical literature for the treatment of osteoporosis. For example, estrogens, calcitonin and bisphosphonates are known to be effective inhibitors of bone resorption.

EP 980250 discloses, inter alia, nutritional, e.g. veterinary, or pharmaceutical compositions, e.g. animal medicines, comprising a plant extract or concentrate of allium and their use for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis. The subject matter of EP 980250 is incorporated by reference to this application.

However, the active constituents of allium responsible for the bone resorption inhibitory effect have not yet been described.

After long and exhaustive testing it has now surprisingly been found that the active constituent of allium responsible for the bone resorption inhibiting effect, may be found in an hydrophilic, ethanolic extract of allium such as *Allium cepa*. The active constituent having a

potent inhibitory effect on bone resorption was identified as a  $\gamma$ -glutamyl-peptide, for example a  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide, further example a  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.

$\gamma$ -glutamyl-peptide, for example a  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide, further example a  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, are hereinafter referred to as active ingredient of the invention. According to the invention, the active ingredient of the invention can be in a concentrate form.

Accordingly, in one aspect the present invention relates to the use of  $\gamma$ -glutamyl-peptide, e.g. in a concentrate form, for example a  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide, further example  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, e.g. in a concentrate form, in the preparation of a medicament or nutritional formulation, e.g. animal medicine or veterinary composition, for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or osteoporosis. In a further aspect, the invention relates to nutritional, e.g. veterinary, or pharmaceutical compositions, e.g. animal medicine, comprising the active ingredient of the invention, e.g. in a concentrate form.

The invention further provides a method for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or osteoporosis, comprising the administration of a medicament or nutritional formulation to a human or an animal, e.g. a mammal, said medicament or nutritional formulation comprising the active ingredient of the invention, e.g. in a concentrate form, in an amount which is effective for inhibiting bone resorption.

In yet a further aspect, the present invention provides for a method of inhibiting bone resorption which method comprises administering to a human or an animal, e.g. a mammal, in need thereof an effective amount of a composition comprising the active ingredient of the invention, e.g. in a concentrate form.

In yet a further aspect the present invention provides for the use of comprising the active ingredient of the invention, e.g. in a concentrate form, in the dietary management of increased bone resorption.

Osteoporosis as used herein includes osteoporosis induced by hormone deficiency (e.g. postmenopausal) and old age, as well as secondary osteoporosis such as osteoporosis secondary to steroid treatment or secondary to malnutrition caused by anorexia nervosa.

The active ingredient of the invention may be isolated from allium, e.g. Allium cepa, e.g. from the whole eatable part of the vegetable, by fractionation, e.g. in vitro bioassay guided fractionation, for example as described hereinbelow. Alternatively, the active ingredient of the invention, in particular the cis-form thereof, may be obtained by full or semi chemical synthesis, for example as readily known to one skilled in the art.

In one aspect, the invention provides the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, e.g. in a concentrate form, obtained by fractionation of an hydrophilic, ethanolic extract of Allium cepa, which fractionation comprises

- (a) obtaining an hydrophilic, ethanolic extract of Allium cepa, hereinafter referred to as fraction A, by using adsorption column chromatography, e.g. Amberlite® XAD-4,
- (b) separating saccharides from fraction A by using reversed-phase medium pressure liquid chromatography (RP-MPLC) to obtain fraction A1,
- (c) further separating saccharides from fraction A1 by using normal-phase medium pressure liquid chromatography (NP-MPLC), e.g. using a mobile phase chosen from
  - (c1) methylethylketone – acetic acid – methanol, e.g. in a ratio of 6:5:3 (v/v),
  - (c2) acetone – water – hydrochloric acid 37%, e.g. in a ratio of 9ml:1ml:1drop,
  - (c3) n-butanole – acetic acid – diethylether – water, e.g. in a ratio of 9:6:3:1 (v/v),
  - (c4) chloroform – methanol – water, e.g. in a ratio of 6.4:5:1,

for example using chloroform – methanol – water 6.4:5:1 as mobile phase, to obtain fraction A1-4,

- (d) further fractionation by semi-preparative reversed-phase HPLC (SP-RP-HPLC), e.g. using as solvent an isocratic water/acetonitrile system buffered with e.g. 0.00625% formic acid to obtain fraction A1-4C.

In a further aspect, the invention provides the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, e.g. in a concentrate form, obtained by in vitro bioassay guided fractionation of an hydrophilic, ethanolic extract of Allium cepa, which bioassay guided fractionation comprises

- (a) obtaining an hydrophilic, ethanolic extract of Allium cepa, hereinafter referred to as fraction A, by using adsorption column chromatography, e.g. Amberlite® XAD-4,

- (b) assessing in vitro the bone resorbing inhibitory activity by using the osteoclast pit assay,
- (c) separating saccharides from fraction A by using reversed-phase medium pressure liquid chromatography (RP-MPLC) to obtain fraction A1,
- (d) assessing in vitro the bone resorbing inhibitory activity by using the osteoclast pit assay,
- (e) further separating saccharides from fraction A1 by using NP-MPLC, e.g. using a mobile phase chosen from
  - (e1) methylethylketone – acetic acid – methanol, e.g. in a ratio of 6:5:3 (v/v),
  - (e2) acetone – water – hydrochloric acid 37%, e.g. in a ratio of 9ml:1ml:1 drop,
  - (e3) n-butanole – acetic acid – diethylether – water, e.g. in a ratio of 9:6:3:1 (v/v),
  - (e4) chloroform – methanol – water, e.g. in a ratio of 6.4:5:1,for example using chloroform – methanol – water 6.4:5:1 as mobile phase, to obtain fraction A1-4,
- (d) assessing in vitro the bone resorbing inhibitory activity by using the osteoclast pit assay,
- (e) further fractionation by semi-preparative reversed-phase HPLC (SP-RP-HPLC), e.g. using as solvent an isocratic water/acetonitrile system buffered with 0.00625% formic acid to obtain fraction A1-4C,
- (f) assessing in vitro the bone resorbing inhibitory activity by using the osteoclast pit assay.

It will be appreciated that the osteoclast pit assay is a well established in vitro model of bone resorption and readily known to one skilled in the art. Briefly, medium containing a sample to be tested, e.g. 30 mg or less of freeze-dried hydrophilic fraction A per ml, is added to osteoclasts of new-born rats settled on ivory slices. After 24 hours of incubation, the tartrate-resistant acid phosphatase positive multi-nucleated cells, i.e. osteoclasts, are counted. Subsequently, the number of resorption pits is determined. Activity is calculated as the ratio of resorption pits per osteoclasts and compared to a negative control, e.g. medium containing 10% fetal bovine serum and to a positive control, e.g.  $10^{-12}$  M calcitonin. For the analysis of statistical significance, the ratios of the treated groups  $\pm$  their respective SEMs are compared to the 95% confidence interval of the SEM of the negative control.

The compound of fraction A1-4C may be analyzed by mass spectroscopy, e.g. using a HPLC-ESI-MS equipment, e.g. using an MS equipped with a quadrupole ion trap (QIT).

Fragmentation may be achieved by colliding the positively charged, ionized molecule with helium gas, e.g. using a collision energy of 35%.

The structure of the compound of fraction A1-4C may be further confirmed by ESI-MS-MS after acid hydrolysis.

Furthermore, the structure of the compound of fraction A1-4C may be confirmed by nuclear magnetic resonance (NMR) spectroscopy, e.g.  $^1\text{H}$ -NNMR,  $^1\text{J}_{\text{CH}}$ -COSY NMR,  $^1\text{H}$ -H-COSY, and/or  $^n\text{J}_{\text{CH}}$ -COSY NMR, e.g. using  $\text{D}_2\text{O}$  as solvent and trimethylsilyl-propansulfonic acid as external standard.

It will be appreciated that such techniques for the analysis of the compound of fraction A1-4C are readily known to one skilled in the art.

As used herein, the term allium refers to the genus allium (latin for garlic, a member of the onion family) and includes for example any member of the botanical species *Allium cepa* (onion), *Allium ascalonicum* (shallot), *Allium ampeloprasum* (leek/great-headed-garlic), *Allium porrum* (leek), *Allium schoenoprasum* (chive), *Allium ursinum* (bear's garlic), *Allium sativum* (garlic) or *Allium fistulosum* (bunching onion). Preferred species are *Allium ascalonicum* (shallot), *Allium porrum* (leek), *Allium cepa* (onion) and *Allium ursinum* (bear's garlic, also known as bear paw garlic), particularly the latter two, whereby *Allium cepa* is particularly preferred. Examples of members of the species *Allium cepa* are common onions (with red or white or yellow skins) or shallots, whereby red or white common onions are preferred.

The extract containing the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, may be used in liquid form, for example in aqueous form, or in solid form, for example in granulate or powder form. Alternatively, the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, may be used as such, e.g. in solid, for example in powder or granulate form, or dissolved or dispersed in a liquid, e.g. in an aqueous liquid.

The amount of the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, to be supplied may vary within wide ranges, depending on i. a. the



desired treatment, subject to be treated, e.g. human or a animal, and his needs. Thus, where the subject to be treated is an adult person (typically of ca. 60 to 75 kg body weight), a satisfactory inhibitory effect on bone resorption may, in general, be obtained with compositions formulated to allow a daily administration from about 20 to about 100 mg/kg, for example from about 40 to about 80 mg/kg of the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, e.g. on a solvent-free basis.

According to the invention, the term "nutritional compositions" refers to nutritional formulations and nutritional products, adapted for humans or animals, e.g. for mammals. The nutritional compositions according to the invention may be in the form of e.g. nutraceuticals, complete formula diet, nutritional or dietary supplements, such as animal feed supplement, functional food, beverage products, meal replacement, or food additives.

Suitable nutritional compositions, e.g. animal feed supplements, comprising the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, represent a further object of the invention. Accordingly, in one aspect the present invention provides a nutritional composition, e.g. an animal feed supplement, comprising:

- (a) the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide,
- (b) a calcium source, and
- (c) at least one energy source selected from the group consisting of carbohydrate, fat and nitrogen sources, and optionally
- (d) Vitamin D.

Regarding component (a), the definitions, preferences and amounts given before for  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide apply. The nutritional compositions of the invention, e.g. animal feed supplements, conveniently comprise an amount of component (a) to allow a daily-administration from about 20 to about 100 mg/kg, for example from about 40 to about 80 mg/kg.

The calcium source (b) may comprise any physiological acceptable inorganic or organic compound containing calcium. Examples are inorganic calcium salts, for example calcium chloride, calcium phosphate, calcium sulfate, calcium oxide, calcium hydroxide or calcium carbonate, or organic calcium components like whole or skim milk powder, calcium caseinate

or calcium salts of organic acids such as calcium citrate, calcium maleate, or mixtures thereof. The use of organic calcium compounds, particularly skim milk powder, calcium caseinate or mixtures thereof, as calcium source (b) is preferred. The amount of calcium component to be supplied may vary within wide ranges. In general, the inventive compositions comprise in one unit dosage from about 100 mg to 1000 mg, preferably 200 mg to 700 mg and most preferred 300 to 600 mg of calcium (on an elemental basis).

The nutritional compositions of the invention, e.g. animal feed supplements, conveniently comprise for example from approximately 1 to 60 % by weight, preferably from approximately 5 to 50 % by weight and most preferred from 10 to 40 % by weight of calcium component (b).

Suitable carbohydrate sources include for example maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and/or sorbit. In these forms the carbohydrates are both energy suppliers and sweeteners. The inventive compositions may contain one or more different carbohydrate sources.

Suitable fat sources include omega-6 polyunsaturated fatty acid sources, omega-3 polyunsaturated fatty acid sources, mono-unsaturated fatty acid sources, medium chain fatty acid sources (i.e. C<sub>6</sub>-C<sub>12</sub>-fatty acids); or mixtures thereof. The above-mentioned fatty acids may be employed in each case in form of the free acid, in mono-, di- or particularly in triglyceride form, or in form of a pharmacological or nutritional acceptable natural source.

Suitable natural sources of omega-6 polyunsaturated fatty acids include vegetable oils such as safflower oil, sunflower oil, soya oil, cotton oil and corn oil. Suitable natural sources of omega-3 polyunsaturated fatty acids include linseed oil and fish oils such as menhaden oil, salmon oil, mackerel oil, tuna oil codliver oil and anchovy oil.

Suitable natural sources of mono-unsaturated fatty acid sources are particularly omega-9 mono-unsaturated fatty acids, for example olives, canola, safflower (hybrids) and sunflower (hybrids).

A preferred fat source comprises triglyceride oils supplying the desired amounts of omega-6 polyunsaturated fatty acids and omega-3 polyunsaturated fatty acids and which are rich in the medium chain fatty acid residues (i.e. residues of C<sub>6</sub>-C<sub>12</sub> fatty acid) and/or mono-

unsaturated fatty acid residues. The inventive compositions, e.g. animal feed supplements, may contain one or more different fat sources.

Examples of suitable nitrogen sources of the inventive nutritional compositions, e.g. animal feed supplements, include sources containing nutritionally acceptable proteins such as soy bean derived proteins; milk proteins such as whey proteins or caseinates; and/or protein hydrolysates; and/or essential amino acids mixtures in free amino acid form or salt form; and/or compounds associated with the synthesis of polyamines, such as arginine, arginine precursors, ornithine and the like, in free amino acid form or salt form.

Preferred nitrogen sources of the nutritional compositions, e.g. animal feed supplements, are

- (i) soy bean derived proteins, which may be employed in the form of soy beans or in the form of any suitable soja extract or concentrate, for example in form of soy flour, dried soy sprouts, soybean milk, or as dried aqueous extract from soybeans; or
- (ii) milk proteins, for example whey derived proteins or caseinates which may be employed for example in the form of whey powder, caseinate salts such as calcium caseinate and/or whole or preferably skim milk powder and/or
- (iii) a mixture of essential amino acids and/or
- (iv) arginine as nitrogen source.

Milk proteins such as whey powder, caseinates, particularly calcium caseinate, and/or skim milk powder are another particularly preferred nitrogen source of the claimed nutritional compositions. The inventive compositions, e.g. animal feed supplements, may contain one or more different nitrogen sources.

The nutritional compositions, e.g. animal feed supplements, comprise for example, from approximately 0.1 % to 98.9 % by weight, preferably from approximately 1 to approximately 95 % by weight, and most preferred from 10 to 90 % by weight of energy source component (c).

The contribution of the nitrogen source, carbohydrate source and fat source to the caloric of the inventive nutritional compositions, e.g. animal feed supplements, may vary within wide ranges. For example, the carbohydrate source provides for 30 to 70 % of the total energy supply, the nitrogen source for 5 to 45% and the fat source for 0.1 to 15 % of the total energy supply of the composition. In preferred compositions of the invention the

carbohydrate source provides for 40 to 60 % of the total energy supply, the nitrogen for 20 to 35 % and the fat source for 3 to 12 % of the total energy supply of the composition.

A preferred energy source (c) of the inventive compositions, e.g. animal feed supplements, comprises  
30 to 70 % of the total energy supply of one or more carbohydrate sources selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and sorbit;  
5 to 45 % of the total energy supply of one or more nitrogen sources selected from the group consisting of soy bean derived proteins, milk proteins, a mixture of essential amino acids and arginine and  
0. 1 to 15 % of the total energy supply of one or more fat sources comprising omega-3- and omega-6-polyunsaturated fatty acids.

A particularly preferred energy source (c) of the inventive compositions comprises  
40 to 60 % of the total energy supply of one or more carbohydrate sources selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and sorbit;  
20 to 35 % of the total energy supply of one or more nitrogen sources selected from the group consisting of soy bean derived proteins, skim milk powder and caseinates; and  
3 to 12 % of the total energy supply of one or more fat sources comprising omega-3- and omega-6-polyunsaturated fatty acids.

The amount of Vitamin D (optional component (d)) to be supplied may vary within wide ranges. In general, the inventive compositions comprise in one unit dosage from about 400 IU to 1000 IU, preferably about 500 IU.

The nutritional formulations of the invention, e.g. animal feed supplements, may comprise other nutritionally acceptable components such as vitamins, minerals, trace elements, fibers (preferably soluble fibers), flavors, preservatives, colorants, sweeteners, emulsifiers and the like.

Examples of vitamins suitable for the incorporation in the composition of the invention, e.g. animal feed supplements, include Vitamin A, Vitamin D, Vitamin E, Vitamin K, Vitamin C, folic acid, thiamin, riboflavin, Vitamin B<sub>6</sub>, Vitamin B<sub>12</sub>, niacin, biotin and panthotenic acid in pharmaceutical or nutritionally acceptable form.

Examples of mineral elements and trace elements suitable for the incorporation in the composition of the invention, e.g. animal feed supplements, include sodium, potassium, phosphorous, magnesium, copper, zinc, iron, selenium, chromium and molybdenum in pharmaceutical or nutritionally acceptable form.

The term soluble fiber as used herein refers to fibers which are able to substantially undergo fermentation in the colon to produce short chain fatty acids. Examples of suitable soluble fibers include agar-agar, alginates, carubin, carrageenan, gum arabic, guar gum, karaya gum, locust bean gum, pectin, tragacanth, or xanthan gum. They may be hydrolysed or not.

Suitable flavors include natural or artificial flavors, for example fruit flavors such as banana, orange, peach, pineapple or raspberry; vegetable flavors; or vanilla, cocoa, chocolate, coffee and the like.

Preferred ingredients of the inventive nutritional compositions, e.g. animal feed supplements, in addition to components (a), (b), (c) and (d) comprise beta-carotene (Vitamin A), Vitamin E, Vitamin C, thiamin, Vitamin B<sub>1</sub>, B<sub>6</sub> and/or B<sub>12</sub>, potassium, magnesium, selenium, zinc, phosphorous and soluble fiber in pharmaceutical or nutritionally acceptable form.

The nutritional compositions, e.g. animal feed supplements, may comprise for example, from approximately 0.1 % to 15 % by weight, preferably from approximately 0.2 to approximately 10 % by weight, and most preferred from 0.5 to 5 % by weight of these additional components other than components (a), (b), (c) and optionally (d).

The inventive nutritional formulations, e.g. animal feed supplements, may be formulated and administered in any form suitable for enteral administration, for example oral administration or tube feeding, e.g. nasal administration. The formulations are conveniently administered in the form of an aqueous liquid. The formulations suitable for enteral application are accordingly preferably in aqueous form or in powder or granulate form, whereby the powder or granulate is conveniently added to water prior to use. For use as tube feeding, the amount of water to be added will i.a. depend on the patient's fluid requirements and condition.

The inventive nutritional compositions, e.g. animal feed supplements, may be in form of a complete formula diet (in liquid or powder form), such that, when used as sole nutrition source essentially all daily caloric, nitrogen, fatty acids, vitamin, mineral and trace element

requirements are met. In general, the daily amount to be supplied to adult persons will lie in the range of 750 to 3500 kcal/day, in particular of 1000 to 2000 kcal/day. However, the inventive nutritional compositions are preferably intended for use as a dietary supplement. The amount of energy supplied by a supplement should not be too excessive, in order not to unnecessarily suppress the patients appetite. The supplement conveniently comprises energy sources in an amount supplying from 50 to 1500 kcal/day, preferably 100 to 900 kcal/day and most preferred 150 to 700 kcal/day.

The nutritional compositions of the invention, e.g. animal feed supplements, which are in liquid form, for example in drink form, or in solid form, for example in granulate or powder form, may be obtained in a manner known *per se*, e.g. by admixing the ingredients and optionally adding water.

The invention further relates to pharmaceutical compositions, e.g. animal medicines or veterinary compositions, in single unit dose form comprising

- (a) the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, and
- (b) a pharmaceutical acceptable carrier, e.g. a carrier physiologically well tolerated by animals.

These pharmaceutical compositions, e.g. veterinary compositions, are compositions for enteral administration, such as oral, nasal or rectal administration. Suitable pharmaceutical compositions may be in liquid form or in solid form and comprise for example, from approximately 0.001 % to 100 % by weight, further example from approximately 0.1 to approximately 50 % by weight, active ingredient (a).

Pharmaceutical compositions, e.g. veterinary compositions, for enteral administration are, for example, those in single unit dose forms, such as dragées, tablets, capsules or sachets. They are prepared in a manner known *per se*, for example by means of conventional mixing, granulating, confectioning, dissolving or lyophilising processes.

For example, pharmaceutical compositions for oral administration can be obtained by combining the active ingredient with solid carriers, optionally granulating a resulting mixture and processing the mixture or granules, if desired or necessary after the addition of suitable excipients, to form tablets or dragée cores.

Suitable carriers are especially fillers, such as sugars, for example lactose, saccharose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tri-calcium phosphate or calcium hydrogen phosphate, and also binders, such as starch pastes using, for example, corn, wheat, rice or potato starch, gelatin, tragacanth, methylcellulose and/or polyvinylpyrrolidone, and, if desired, disintegrators, such as the above-mentioned starches, and also carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Excipients are especially flow-conditioners and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol. Dragée cores are provided with suitable, optionally enteric, coatings, there being used *inter alia* concentrated sugar solutions which may contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents or solvent mixtures or, for the preparation of enteric coatings, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate. Dyes or pigments may be added to the tablets or dragée coatings, for example for identification purposes or to indicate different doses of active ingredient.

Other orally administrable pharmaceutical compositions are hard gelatin capsules and also soft, sealed capsules consisting of gelatin and a plasticiser, such as glycerol or sorbitol. The hard gelatin capsules may comprise the active ingredient in the form of granules, for example in admixture with fillers, such as lactose, binders, such as starches, and/or glidants, such as talc or magnesium stearate, and, if desired, stabilisers. In soft capsules the active ingredient is preferably dissolved or suspended in suitable liquids, such as fatty oils, paraffin oil or liquid polyethylene glycols, it is likewise being possible to add stabilisers.

Suitable rectally administrable pharmaceutical compositions are, for example, suppositories that consist of a combination of the active ingredient with a suppository base material. Suitable suppository base materials are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylen glycols or higher alkanols. It is also possible to use gelatin rectal capsules which comprise a combination of the active ingredient with a base material. Suitable base materials are, for example, liquid triglycerides, polyethylenglycols or paraffin hydrocarbons.

The inhibitory effect of the active ingredient of the invention, e.g.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide on bone resorption, may be assessed by an *in vitro* assay, e.g.

as described hereinabove, in which ivory slices, onto which freshly isolated osteoclasts have been settled, are incubated with a medium containing the extract or concentrate to be tested. The inhibitory effect on osteoclasts is assessed by counting the osteoclast resorption pits on the ivory slice.

The following Examples illustrate the invention.

#### Example 1: Bioassay guided fractionation

1.1 To obtain a hydrophilic onion fraction, fraction A, adsorption column chromatography is used. The column is slurry-filled using aqueous 85% ethanol. After filling, the stationary phase (Amberlite XAD-4) is stepwise washed with 400 ml ethanol, 500 ml aqueous 85% ethanol, 2500 ml water and 500 ml aqueous 85% ethanol. After washing, the stationary phase is conditioned with the first solvent used for separation, i.e. aqueous ethanol 15% (v/v). 38.10 g of dry onion powder are dissolved in 600 ml aqueous ethanol 15% (v/v) and heated (60°C) for 30 min under constant stirring. After cooling to room temperature, the turbid solution is centrifuged for 20 min at 7000 rpm and the supernatant is subjected to fractionation. The residue is discarded.

The pooled 15% aqueous fractions devoid of flavonoids, e.g. quercetin, and the 85% aqueous ethanolic fraction containing the flavonoids are reduced under vacuo at 40°C and freeze-dried. The water fraction is discarded.

1.2 In order to separate saccharides such as fructose, glucose and sucrose from the active constituents of fraction A, preliminary reversed-phase high performance thin layer chromatography (RP-HPTLC) experiments, using 5% aqueous methanol as mobile phase, was performed. The method is upscaled on a reversed-phase medium pressure liquid chromatography (RP-MPLC) column and 1.0g samples of fraction A are subjected to separation. Fractionation monitoring is performed by TLC using anisaldehyde reagent for detection. In order to recover as much starting material as possible, after each run, the column is thoroughly washed with methanol.

In order to isolate the bone resorbing inhibitory compound(s) of the hydrophilic onion fraction, fraction A, a bioassay guided fractionation is performed. To assess in vitro the bone resorbing inhibitory activity, the osteoclast pit assay is used. Medium containing 30 mg or



less of freeze-dried fraction per ml is added to osteoclasts of new-born rats settled on ivory slices. After 24 hours of incubation, the tartrate-resistant acid phosphatase positive multi-nucleated cells, i.e. osteoclasts, are counted. Subsequently, the number of resorption pits is determined. Activity is calculated as the ratio of resorption pits per osteoclasts and compared to a negative control, i.e. medium containing 10% fetal bovine serum and to a positive control, i.e.  $10^{-12}$  M calcitonin. For the analysis of statistical significance, the ratios of the treated groups  $\pm$  their respective SEMs are compared to the 95% confidence interval of the SEM of the negative control.

a) TLC-screening and yields of the RP-MPLC fractionations.

The fraction containing the saccharides is named "fraction A2", the fraction devoid of saccharides "fraction A1".

Fraction	Yields (g)	Yields (%)
A1	4.75	36.5
A2	7.18	55.2
Total	11.93	91.7

b) Biological results of the RP-MPLC fractionations

Doses are given in mg per ml and results as resorption pits per tartrate resistant acid phosphatase positive (TRAP+) cells  $\pm$  SEM.

Sample	Pits/TRAP+ cells $\pm$ SEM
Neg. control	0.489 $\pm$ 0.128
CT $10^{-12}$ M	0.061 $\pm$ 0.063
30 mg fraction A	0.021 $\pm$ 0.021
12 mg fraction A1	0.243 $\pm$ 0.113
Neg. control	0.957 $\pm$ 0.327
24 mg fraction A1	0.144 $\pm$ 0.066
Neg. control	0.251 $\pm$ 0.071
30 mg fraction A	0.015 $\pm$ 0.009
30 mg fraction A1	0.035 $\pm$ 0.016
Neg. control	1.210 $\pm$ 0.254
30 mg fraction A1	0.020 $\pm$ 0.009
30 mg fraction A2	0.399 $\pm$ 0.133
30 mg fraction A2	0.384 $\pm$ 0.124
30 mg fraction A	0.115 $\pm$ 0.048

## c) Discussion and conclusion

Fraction A1 does not inhibit significantly osteoclast activity tested at the 1-fold proportional amount (12 mg/ml). However, the double dose (24 mg/ml) decreases osteoclast activity significantly to a pits/cells ratio of 0.144 (-40% compared to 12 mg/ml) and at 30 mg/ml even stronger inhibitions of osteoclast activity, i.e. 0.015 and 0.020 pits/cells (-90% compared to 12 mg/ml) can be measured. It is concluded that fraction A1 contains compounds inhibiting osteoclast activity.

The slight osteoclast activity inhibition by fraction A2 may be explained by the presence of small amounts of compounds of fraction A1.

Fraction A1 is chosen to continue the bioassay guided fractionation, fraction A2 is discarded. Because the bone resorbing inhibitory compound eluted still with the saccharides, an additional fractionation to separate the saccharides from the active compound(s) is performed.

1.3 Four different mobile phases for NP-TLC of polar compounds or sugars are tested in order to select the appropriate method for the next preparative separation. To evaluate the separation efficiency of the system, fraction A1 and the saccharides fructose, glucose and sucrose are used as samples:

- (a) methylethylketone – acetic acid – methanol, 6:5:3 (v/v),
- (b) acetone – water – hydrochloric acid 37%, 9ml:1ml:1drop,
- (c) n-butanole – acetic acid – diethylether – water, 9:6:3:1 (v/v),
- (d) chloroform – methanol – water, 6.4:5:1.

All TLCs are performed on NP-TLC and sprayed with Anisaldehyde reagent for visualization. There are no remarkable qualitative differences between the mobile phases used. Mobile phase (d) was chosen to perform the next preparative separation step.

The NP-TLC system described above is upscaled to a NP-MPLC column and samples of 400 mg of fraction A1 are subjected to fractionation. Fractionation monitoring is again performed by NP-TLC. When no more spots are observed on TLC, the system is thoroughly washed with 70% aqueous methanol (v/v).

## a) TLC-screening and yields of the NP-MPLC fractionations

Fraction	Yields(mg)	Yields (%)
A1-1	0.562	28.1

A1-2	0.578	28.9
A1-3	0.352	17.6
A1-4	0.146	7.3
Total	1.638	81.9

b) Biological results of the NP-MPLC fractionations

Doses are given in mg per ml and results as resorption pits per tartrate resistant acid phosphatase positive (TRAP+) cells  $\pm$  SEM. In order to counteract losses during the fractionation, except for fraction A1-2, all fractions are tested at the one-, two- and three-fold proportional amount of their respective yields compared to fraction A.

Sample	Pits/TRAP+ cells $\pm$ SEM
Neg.control	0.990 $\pm$ 0.168
4.57 mg fraction A1-1	0.642 $\pm$ 0.207
9.88 mg fraction A1-1	0.809 $\pm$ 0.342
13.7 mg fraction A1-1	0.691 $\pm$ 0.147
Neg.control	0.957 $\pm$ 0.327
5.28 mg fraction A1-2	0.835 $\pm$ 0.231
15.8 mg fraction A1-2	1.000 $\pm$ 0.230
Neg.control	0.818 $\pm$ 0.141
2.15 mg fraction A1-3	0.631 $\pm$ 0.186
4.64 mg fraction A1-3	0.569 $\pm$ 0.277
6.44 mg fraction A1-3	1.425 $\pm$ 0.396
Neg.control	1.419 $\pm$ 0.364
1.48 mg fraction A1-4	2.158 $\pm$ 0.888
3.12 mg fraction A1-4	3.636 $\pm$ 1.262
4.76 mg fraction A1-4	0.010 $\pm$ 0.010
4.76 mg fraction A1-4	0.096 $\pm$ 0.026

c) Discussion and conclusion

In fractions A1-1, A1-2 and A1-3 no inhibition is measurable. The pits per cell ratio of all these fractions are located inside the 95% confidence interval of the SEM of the negative control. Fraction A1-4, completely free of sugars, shows a significant osteoclast activity inhibition at the three-fold dose. The apparent stimulation of the cell activity at the one- and

two-fold dose may be explained by a strong decrease in cell number. Fraction A1-4 is chosen for further fractionation.

#### 1.4 Fractionation of fraction A1-4 by semi-preparative reversed-phase HPLC (SP-RP-HPLC)

Fraction A1-4 is further fractionated with SP-RP-HPLC into four fractions A1-4A, A1-4B, A1-4C and A1-4D using as solvent an isocratic water/acetonitrile system buffered with 0.00625% formic acid. Fraction A1-4B contains two minor compounds of fraction A1-4, fraction A1-4C consists of the most predominant compound of fraction A1-4, fractions A1-4A and A1-4D are the prerun and afterrun. Fractions A1-4A and A1-4D are pooled together for the further tests. On the whole, 8.125 mg of fraction A1-4 are separated in 65 single HPLC runs applying 0.125 mg in each run and pooling the fractions. Fractionation is performed manually by switching a tap at the outlet of the HPLC equipment.

##### a) Yields of the SP-RP-HPLC fractionations

Fraction	Yields (mg)	Yields (%)
A1-4A	3.7	35.2
A1-4B	1.3	12.5
A1-4C	1.6	15.2
A1-4D	3.9	37.1
Total A1-4A – A1-4D	10.5	100.00

##### b) Biological results of the SP-RP-HPLC fractionations

Sample	Pits/TRAP+ cells $\pm$ SEM
Neg. control	$0.739 \pm 0.138$
CT $10^{-12}$ M	$0.116 \pm 0.030$
2.28 mg A1-4	$0.193 \pm 0.055$
2.53 mg A1-4A+D	$0.432 \pm 0.184$
0.43 mg A1-4B	$0.718 \pm 0.208$
0.53 mg A1-4C	$0.325 \pm 0.108$

##### c) Discussion and conclusion

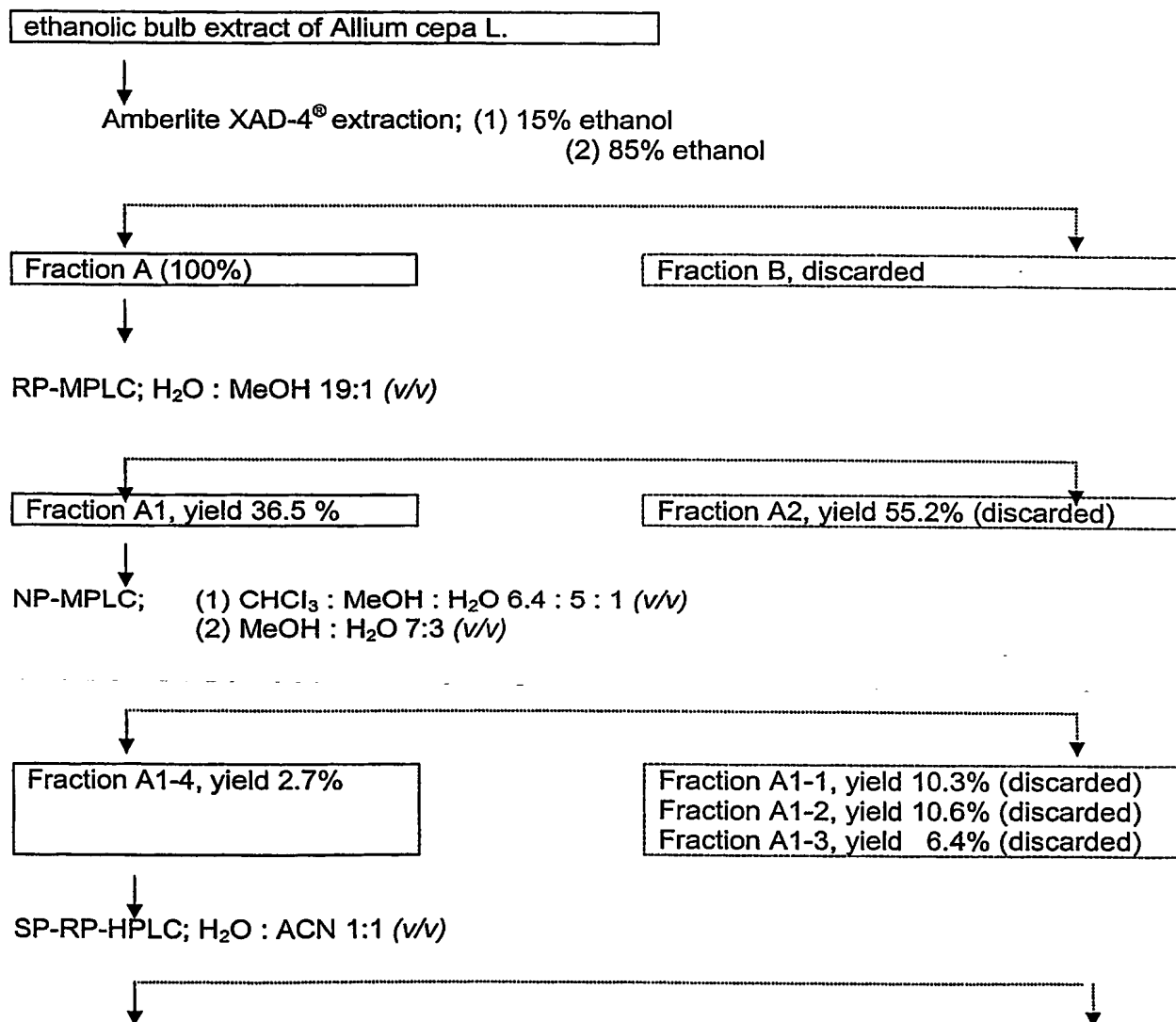
Fraction A1-4 at a three-fold proportional dose corresponding to fraction A1 and fraction A1-4C significantly ( $p < 0.5$ ) inhibit bone resorption. Fraction A1-4C inhibits osteoclast activity

similar to fraction A1-4, indicating that A1-4C is the sole active compound of fraction A1-4. The isolation of A1-4C is repeated to recover sufficient amounts for structure elucidation experiments.

Summary: An ethanolic bulb extract is purified by sequential use of four different chromatographic systems - Amberlite XAD-4 extraction; RP-MPLC; NP-MPLC; and semi-preparative RP-HPLC. Resulting fraction A1-4C consists of only one compound inhibiting osteoclast activity.

The following graph gives an overview of the bioassay-guided isolation leading to fraction A1-4C:

Bioassay-guided isolation: Overview



Fraction A1-4C, yield 0.40%

Fraction A1-4A, yield 0.94% (discard.)  
Fraction A1-4B, yield 0.33% (discard.)  
Fraction A1-4D, yield 1.01% (discard.)

### Example 2: Structure elucidation experiments

#### 2.1 RP-HPLC-ESI-MS<sup>2</sup> of A1-4C

Mass spectroscopical analysis is performed using HPLC-ESI-MS equipment to obtain first structural information concerning the structure of A1-4C. The MS is equipped with a quadrupole ion trap. Fragmentation is achieved by colliding the positively charged, ionized molecule with helium gas using a collision energy of 35%.

The positively charged parent ion of A1-4C is 307 m/z [m+H]<sup>+</sup>. Literature search revealed the existence of an onion compound of identical mass, namely  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide (g-GPeCSO). Further fragmentation is performed to see whether the resulting fragmentation pattern can be brought into line with this compound.

#### 2.2 ESI-MS of A1-4C after acid hydrolysis

The results obtained by ESI-MS further support the assumption that fraction A1-4C is g-GPeCSO and are not contradictory to the previously established claim after the HPLC-ESI-MS experiments.

#### 2.3 Nuclear magnetic resonance experiments of A1-4C

Measurements are performed in D<sub>2</sub>O using trimethylsilyl-propansulfonic acid as external standard.

The compound of fraction A1-4C can be identified by NMR spectroscopy as  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide (g-GPeCSO), confirming the preceeding HPLC-MS experiments.

### Example 3:

The following is an example of a suitable composition of an inventive Supplement in powder form.

#### Supplement in Powder Form (1 portion)

Content	65.0	g
Extract <sup>1)</sup>	14.5	g

<b>Protein</b>		20.0	g
including	- Ca-caseinate protein	8.7	g
	- skim milk powder	11.0	g
<b>Fat</b>		2.8	g
including	- omega-6 polyunsaturated acids	1.3	g
	- omega-3 polyunsaturated acids	0.03	g
<b>Carbohydrates</b>	(including inventive extract)	31.0	g
including	- lactose	16.5	g
	-maltodextrin	3.5	g
<b>Fiber (soluble)</b>		5.0	g
<b>Further ingredients</b>		3.0	g
including	-Na	230	mg
	-K	500	mg
	-Ca	600	mg
	-Mg	90	mg
	-P	430	mg
	-Cl	350	mg
	-Zn	150	mg
	-Retinol (vitamin A)	0.3	mg
	-Calciferol (vitamin D)	5.0	mcg
	-Tocopherol (vitamin E)	3.0	mg
	-Phylloquinone (vitamin K1)	30.0	mcg
	-Thiamin (vitamin B1)	0.4	mg
	-Riboflavin (vitamin B1)	0.5	mg
	-Pyridoxine (vitamin B6)	0.8	mg
	-Cyanocobalamin (vitamin B12)	0.8	mcg
	-Ascorbic acid (vitamin C)	20.0	mg
	-Biotin	50.0	mcg
	-Folic acid	120.0	mcg
	-Niacinamide	5.0	mg
	-Panthothenic acid	2.0	mg
<b>Energy value</b>		229	kcal

<sup>1)</sup> Fraction A1-4C of Example 1 comprising  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.

The above supplement may be mixed with water and taken in appropriate concentration between meals, e.g. between 2 to 4 times daily.

CLAIMS

1. Use of  $\gamma$ -glutamyl-peptide in the preparation of a medicament or nutritional formulation for humans or animals for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption.
2. Method of inhibiting bone resorption comprising administering to a human or animal in need thereof an effective amount of  $\gamma$ -glutamyl-peptide.
3. Method of treating or preventing a disease or condition which is characterized by increased bone resorption comprising administering to a human or animal in need thereof an effective amount of  $\gamma$ -glutamyl-peptide.
4. Use of  $\gamma$ -glutamyl-peptide in the dietary management of increased bone resorption.
5. The use or method of any preceding claim wherein the  $\gamma$ -glutamyl-peptide is  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamy-alkenyl-cysteine sulfoxide.
6. The use or method of claim 5 wherein the  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide is  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.
7. The use or method of any one of claims 1, 3, 5 or 6 wherein the disease or condition which is characterized by increased bone resorption, is Paget's disease, tumor-induced bone disease or osteoporosis.
8. A nutritional composition comprising  $\gamma$ -glutamyl-peptide and a nutritionally acceptable carrier.
9. The nutritional composition of claim 8 wherein the  $\gamma$ -glutamyl-peptide is  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamy-alkenyl-cysteine sulfoxide.
10. The nutritional composition of claim 9 wherein the  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide is  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.



11. The nutritional composition of any one of claims 8 to 10 further comprising
  - (a) a calcium source,
  - (b) at least one energy source selected from the group consisting of carbohydrate, fat and nitrogen sources, and optionally
  - (c) Vitamin D.
12. The nutritional composition of claim 11, wherein the calcium source (a) is an organic calcium salt.
13. The nutritional composition of claim 11 or 12, wherein the carbohydrate source of component (b) is selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit, sorbit, and mixtures thereof.
14. The nutritional composition of any one of claims 11 to 13, wherein the fat source of component (b) is selected from the group consisting of omega-6 polyunsaturated fatty acid sources, omega-3 polyunsaturated fatty acid sources, mono-unsaturated fatty acid sources, C<sub>6</sub>-C<sub>12</sub>- fatty acid sources, and mixtures thereof.
15. The nutritional composition of any one of claims 11 to 14, wherein the nitrogen source of component (b) is selected from the group consisting of soy bean derived proteins; milk proteins, protein hydrolysates, a mixture of essential amino acids and arginine, and mixtures thereof.
16. The nutritional composition of any one of claims 11 to 15, wherein the carbohydrate source provides for 30 to 70 %, the nitrogen source for 5 to 40 %, and the fat source for 0.01 to 5 % of the total energy supply of the composition.
17. The nutritional composition of any one of claims 11 to 16 comprising from 3 to 25 % by weight of component (a), from 5 to 50 % by weight of component (b) and from 1 to 95 % by weight of component (c), based on the total weight of the nutritional composition.
18. The nutritional composition of any one of claims 8 to 17 further comprising 0.2 to 10 % by weight of other nutritionally acceptable components chosen from vitamins, minerals, trace elements, fibers, flavors, preservatives, colorants, sweeteners and emulsifiers.

- 19.. The nutritional composition of any one of claims 8 to 18 in the form of a dietary supplement providing from 50 to 1500 kcal/day, or in the form of an animal feed supplement.
20. The nutritional composition of any one of claims 8 to 19 in liquid form.
21. The nutritional composition of any one of claims 8 to 20 in granulate or powder form.
22. A pharmaceutical composition in single unit dose form, comprising  $\gamma$ -glutamyl-peptide and a pharmaceutically acceptable carrier.
23. The pharmaceutical composition of claim 22 wherein the  $\gamma$ -glutamyl-peptide is  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamy-alkenyl-cysteine sulfoxide.
24. The pharmaceutical composition of claim 23 wherein the  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide is  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.
25. The pharmaceutical composition of any one of claims 22 to 24 for enteral administration in the form of a dragée, tablet, capsule, sachet or suppository.
26. The pharmaceutical composition of any one of claims 22 to 25 in the form of a veterinary composition.
27.  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide obtained by fractionation of an hydrophilic, ethanolic extract of *Allium cepa*, which fractionation comprises
- (a) obtaining an hydrophilic, ethanolic extract of *Allium cepa*, hereinafter referred to as fraction A, by using adsorption column chromatography,
  - (b) separating saccharides from fraction A by using reversed-phase medium pressure liquid chromatography (RP-MPLC) to obtain fraction A1
  - (c) further separating saccharides from fraction A1 by NP-MPLC using chloroform – methanol – water 6.4:5:1 as mobile phase, to obtain fraction A1-4,
  - (d) further fractionation by semi-preparative reversed-phase HPLC (SP-RP-HPLC) using as solvent an isocratic water/acetonitrile system buffered with e.g. 0.00625% formic acid to obtain fraction A1-4C.

28. Process for producing a veterinary composition for the treatment or prophylaxis of a disease or condition in animal which is characterized by increased bone resorption or for the management of increased bone resorption in animal comprising homogenizing a mixture of one or more carriers that are physiologically acceptable to animals and an effective amount of a  $\gamma$ -glutamyl-peptide.

29. The process of claim 28 wherein the  $\gamma$ -glutamyl-peptide is  $\gamma$ -glutamyl-alkyl-cysteine sulfoxide or  $\gamma$ -glutamy-alkenyl-cysteine sulfoxide.

30. The process of claim 29 wherein the  $\gamma$ -glutamyl-alkenyl-cysteine sulfoxide is  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.

ABSTRACT

The present invention concerns the use of  $\gamma$ -glutamyl-peptide, for example  $\gamma$ -L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide, in the treatment or prevention of diseases or conditions which are characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or osteoporosis.

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